Purification and immunological properties of vanadate sensitive Mg:ATPase from plasma membrane of maize roots*

An-Fei C. Hsu, David Brauer, Donna Lu, Stacey Rodenbach and Shu-I Tu

U.S. Department of Agriculture, Eastern Regional Research Center, East Mermaid Lane, Philadelphia, PA 19118 (U.S.A.)

The vanadate-sensitive ATPase from maize (*Zea mays* L.) root plasma membrane was partially purified from KI-washed microsomes by discontinuous sucrose gradient centritugation followed by deoxycholate (DOC) treatment, L-L-lysophosphatidylcholine (lyso-PC) solubilization and glycerol gradient centrifugation. The plasma membrane preparation had a relatively high specific activity of ATPase activity (182 μmol P_i/h (mg proteins). During lyso-PC solubilization, the presence of Mg:ATP and vanadate substantially improved the recovery of active ATPase. The 100-kDa polypeptide of the plasma membrane ATPase was isolated by sodium dodecyl sulfate polyacryamide gel electrophoresis (SDS-PAGE) and used to raise a specific polyclonal antiservm. The addition of antiserum to the lyso-PC solubilized plasma membrane resulted in a loss of 90% of the total ATPase activity from the supernatant fraction after centritugation. The antiserum also inhibited the proton transport activity of reconstituted plasma membrane vesicles. Western blot experiments showed that a single band of 100 kDa reacted with the anti-ATPase antibodies. Enzyme-linked immunosorbant assay (ELISA) analysis indicated that the antiserum exhibited specificity to plasma membrane as compared to other subcellular membranes. Thus, the antiserum obtained in this study is specific for the 100-kDa polypeptide of the plasma membrane ATPase.

Key words: ATPase; solubilization; antiserum; corn roots; immunological properties

Introduction

The plasma membrane ATPase in higher plants has received special attention because of its function in the energy-dependent transport of protons from the cytoplasm to the cell exterior [1], its involvement in plant growth and its response to growth regulators [2]. The plasma membrane H^+-ATPase has been partially purified from various plants [3—6] and fungal sources [7]. Regardless of the employed purification methods, plasma membrane H^+-ATPase has similar physical and chemical properties including sensitivity to various inhibitors, pH optimum and K_m for Mg:ATP. Relatively pure plasma membrane H^+-ATPase has been prepared from oat, tomato and corn roots [3—9]. In all of these reports, the ATPase existed as an oligomer of a 100-kDa polypeptide [7]. Therefore, the exact number of 100-kDa peptides needed for catalytic activity remains unknown. Nagao et al. [10] purified H^+-ATPase from corn root plasma membrane by solubilization with lysolecithin and glycerol gradient centrifugation. The purified enzyme showed a relatively high specific activity (4.7 μmol P_i/min (mg protein). A polyclonal antibody was raised against this purified ATPase and was able to in-

Correspondence to: Dr. An-Fei C. Hsu, Plant and Soil Biophysics Research Unit, U.S. Department of Agriculture, Eastern Regional Research Center, 600 E. Mermaid Lane, Philadelphia, PA 19118, U.S.A.

Abbreviations: ABTS, 2,2′-azino-bis-3-ethyl benzthiazoline sulfate; AO, acridine orange; ATP, adenosine triphosphate; DTT, dithiothreitol; DOC, deoxycholate; EDTA, ethylenediamine tetraacetate; EGTA, ethyleneglycerolbis-(*b*-aminoethyl ether) *N,N′*-tetraacetic acid; ELISA, enzyme-linked immunosorbant assay; GAR, Gold Anti-Rabbit; HRP, horseradish peroxidase; lyso-PC, L-L-lysophosphatidylcholine; MES, 2(*N*-morpholino)ethanesulfonic acid; PBS, phosphate buffered saline; P_i, inorganic phosphate; PMSF, phenylmethyl sulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Na_3VO_4, sodium vanadate; TBS, Tris buffered saline.

*Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

hibit most of the enzyme activity. DuPont et al. [11] utilized SDS-PAGE to demonstrate that antibodies could cross react with plasma membrane ATPase from different plant sources. Although in many studies antibodies against plasma membrane ATPase have been used, a direct demonstration of the inhibition of proton transport by the antibody has not yet been reported. In addition, the immunological properties of the plasma membrane ATPase antibodies such as specificity to various membranes were not characterized. In this report, we describe some of the immunological properties of the antibody against corn root plasma membrane ATPase and demonstrate that the antibody is able to inhibit both proton pumping and ATP hydrolysis activities of the ATPase.

Materials and Methods

Purification of vanadate sensitive ATPase

Crude microsomal pellets washed with 0.25 M KI were prepared from 3-day-old roots of maize seedlings (c.v. WF9 × MO 17) as previously described [12]. A plasma membrane-enriched fraction was isolated by pelleting through a sucrose gradient [8]. The membrane fraction was resuspended in 0.15% (w/v) DOC in ice cold buffer containing 10 mM Tris (pH 7.5), 1 mM PMSF and 1.5 μg/ml of chymostatin. The mixture was centrifuged at 100 000 × *g* (1 h, 4°C), and the pellet was then washed with the same buffer without DOC. The membrane fraction was then resuspended in ATPase solubilization buffer (containing 1 mM ATP, 1 mM $MgSO_4$, 0.1 mM Na_3VO_4, 2 μg/ml chymostatin and 15 mg/ml lyso-PC titrated to pH 7.5 with Tris) and then incubated at room temperature for 15 min with occasional agitation. The resulting mixture was centrifuged at 30 000 × *g* for 10 min at 10°C to remove the membranes. The supernatant fraction was adjusted to 0.2% DOC, layered over 12 ml of a linear glycerol gradient 10—40%, (w/v) in a buffer containing 2 mM ATP, 2 mM EDTA, 2 μg/ml chymostatin, 0.2% DOC and 1 mM DTT, and adjusted to pH 6.8 with Tris. After centrifigation at 31 000 rev./min for 18 h at 4°C in a Beckman SW 40 rotor, the gradient was fractionated into 0.6-ml aliquots and assayed for vanadate sensitive ATPase activity (see below).

Other methods

ATPase activity was assayed at 35°C as described by Addison and Scarborough [13] with minor modifications. The reaction was carried out at 35°C for 35 min at pH 6.5 in a 100 μl volume containing 5 mM ATP (Tris salt), 40 mM Tris-MES, 5 mM $MgSO_4$, 50 mM KCl or KNO_3, 1 mM EGTA and 5 mM NaN_3. The reaction was terminated by the addition of 100 μl of 5% (w/v) SDS. The amount of phosphate released was determined by the method of Ames [14].

Proton pumping activity was measured by the initial rate of change of AO absorbance at 493 nm as previously described [12]. Typically, 200 μl of vesicles were diluted to 2 ml with a solution containing 17.5 mM MES-Tris (pH 6.45), 2.5 mM $MgSO_4$, 1 mM EGTA, 7.5 μM AO and 50 mM KNO_3. After equilibrating at room temperature for 10 min, the reaction was initiated by the addition of 20 μl of 0.2 M ATP (pH adjusted to 6.45 with Tris).

Protein content was determined by a modified Lowry [15]. SDS-PAGE was performed on 7.5% Gels with 6% stacking gels and stained with Coomassie Blue as described by Addison and Scarborough [13]. Plasma membrane vesicles were reconstituted by the method of Brauer et al. [12]. Typically, 690 μl of membranes (1.5 mg of protein) and 10 mg of asolectin in 250 μl reconstitution buffer (containing 10 mM MES-Tris, pH 6.45, 50 mM K-acetate and 5 mM NaN_3) were combined and dispersed by the addition of 10% (w/v) DOC to a final concentration of 0.6% (w/v). The detergent was then removed by passage through a G-150 column. Reconstituted proteoliposomes were recovered in the cloudy void volume and used to assay H^+-transport activity and ATPase activity with or without the antiserum.

Production of antibody

The production of antiserum against the ATPase was performed by the Pocono Rabbit Farm and Laboratory (Canadensis, PA). The band corresponding to the 100-kDa polypeptide was excised (50 μg of protein) from the polyacrylamide gel, crushed in a motor and pestle, mixed with Freund's complete adjuvant and injected intramuscularly at multiple loci in rabbits. A second injection with the same amount of antigen was ad-

ministered 1 week later. Serum samples were collected 1 week after the booster injection. Further injections containing the same amount of antigen were repeated 3 times at 1-month intervals. Serum samples were collected 1 week after each additional injection and ELISA was performed to determine the antiserum titer.

ELISA and Western blotting method

The assay was performed in a 96-well microtiter plate (Linbro/Titertek, McLean, VA) using a previously described method [16]. The wells were coated with different dilutions of membrane proteins and incubated for 2 h at room temperature. The plates were then washed extensively with PBS containing 0.1% (w/v) Tween 20. After incubating for another hour in PBS containing 1% (w/v) BSA to block nonspecific binding, 100 μl of rabbit antiserum at 1:500 dilution was added to each well and incubated at 37°C for 2 h. Unreacted antibody was removed by washing several times with PBS. Horseradish peroxidase (HRP) conjugated with goat antirabbit IgG (Bio-Rad) was added at a 1:500 dilution. The plates were incubated at 37°C for 2 h and then washed with PBS. Color development proceeded for 15 min following the addition of the peroxidase substrate, ABTS (1 mM). Absorbance at 415 nm was obtained by ELISA reader. Control experiments were performed the same as above except that zero day (pre-immune) rabbit serum replaced the antiserum.

The Western blotting method was performed at room temperature by using a standard Bio-Rad vertical Trans-Bolt cell (model 250). Gels after SDS-PAGE were transferred to nitrocellulose paper in Tris-Glycine buffer (containing 25 mM Tris and 192 mM glycine, pH 8.3) overnight at 30 V. After blotting, membranes were be stained either by Amido black or immunno-blot reagents. [16]

Determination of ATPase activity following immunoprecipitation

The plasma membrane-enriched fraction was solubilized with lyso-PC (15 mg/ml protein) as described above. Different amounts of ATPase antiserum (1:500 dilution in TBS buffer, containing 20 mM Tris and 150 mM NaCl, pH 7.5) were added to the solubilized plasma membrane. After incubating at 37°C for 2 h, an aliquot of anti-rabbit goat IgG linked to Sepharose-4B (Bio-Rad) beads was added. The mixture was further incubated at 37°C for 2 h, and then centrifuged at 20 000 $\times$ g for 10 min. The ATPase activity in the supernatant fraction was then assayed as described above. The background ATPase activity in the immunoprecipitated pellet was assayed by using zero day (preimmune) serum. Data are the net ATPase activity after subtracting the background ATPase value. All the experiments were carried out in at least duplicate trials, and the experimental variation was less than 5%.

Determination of proton transport and ATPase activity following immunotitration

The reconstituted plasma membrane (80 μg of protein in 125 μl of reconstitution buffer) was mixed with various concentrations of antiserum or preimmune serum to a final volume of 250 μl, (by adding the TBS buffer). The mixture was incubated at room temperature for 2 h, then 200-μl aliquots were assayed for proton transport by the AO method as described above. When proton pumping reached a steady state, 100-μl aliquots from the reaction mixture were assayed for phosphate released by the Malachite-Green method [17]. All values shown for immune serum have been corrected (by subtraction) for the reaction with preimmune serum. The inhibition values using preimmune serum were always less than 10% of the values using immune serum.

Immuno-Gold staining

The protocol for immunodetection was performed using Bio-Rad's Anti-Rabbit (GAR) Gold Conjugate Immuno Blot Kit [18]. After Western blotting transfer, membranes were incubated with TBS buffer for 15 min, then incubated an additional 2 h with blocking buffer (1% BSA in TBS buffer). The membranes were washed several times with TTBS buffer (0.5% Tween-20 in TBS buffer), then further incubated with antiserum (1:500 dilution in TBS buffer) for 2 h at room temperature. After washing the membranes with TTBS for 5 min with gentle agitation, the GAR gold conjugate solution was added to completely cover the membrane. Red dots usually appeared after 1 h. If the GAR gold reagents failed to stain the membrane

within 1 h, an overnight incubation was used to determine whether a visible red color resulted.

Results

Solubilization and partial purification of plasma membrane ATPase

KI-washed microsomes were chosen as the source for obtaining the vanadate-sensitive plasma membrane ATPase, because these vesicles are enriched in plasma membrane ATPase [3]. The KI-washed microsomes were layered over a 34% sucrose pad and crude plasma membrane was obtained from the pellet. To further purify the plasma membrane ATPase, the pellets were treated with various concentrations of DOC. At 0.2% DOC (w/v), ATPase activity was obtained with the highest recovery. Increasing the DOC concentration beyond 0.2% resulted in a loss of ATPase activity. At DOC concentrations less than 0.1%, significant levels of contaminating proteins remained in the pellet. Proteins in the DOC-treated pellet were effectively solubilized by lyso-PC, but the recovery of ATPase activity was dependent on the presence of ATP, Mg^{2+} and vanadate. As shown in Fig. 1, the addition of ATP, Mg^{2+} and vanadate was needed to maintain ATPase activity during lyso-PC solubilization. The addition of Mg^{2+} and vanadate alone slightly increased ATPase activity, but not as much as the addition of ATP, Mg^{2+} and vanadate or Mg^{2+} and vanadate. Under the conditions shown in Fig. 1, SDS-PAGE did not reveal significant differences in the staining intensity of lyso-PC solubilized polypeptides (data not shown). Therefore, the effects of ATP, Mg^{2+} and vanadate were not on the solubilization of the enzyme, but rather on its stability. Lyso-PC did not effectively solubilize ATPase activity from the DOC treated pellet if the buffer washing step between DOC and lyso-PC treatment was omitted (data not shown).

The solubilized membrane fraction was then centrifuged on a linear glycerol gradient (10—40%). After centrifugation, proteins separated as three major peaks along the gradient (Fig. 2). The vanadate-sensitive ATPase activity appeared as one peak located near the top of the gradient. Lipid analysis of each fraction revealed

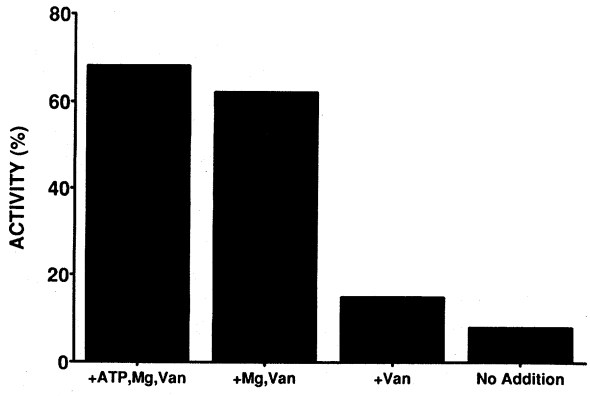

Fig. 1. Effect of Mg^{2+}, ATP and vanadate on the recovery of ATPase activity during lyso-PC solubilization: Plasma membrane were resuspended in lyso-PC solubilization buffer containing lyso-PC and chymostatin, and either 1 mM vanadate, 2 mM Mg^{2+} plus 1 mM vanadate or 2 mM Mg^{2+} plus ATP plus 1 mM vanadate. After centrifugation, the supernatant fraction was tested for ATPase activity and protein concentration was determined as described in Materials and Methods. The 100% activity was the ATPase activity before lyso-PC solubilization. (100% activity of ATPase = 45 μmol P_i h^{-1} mg (prot.)$^{-1}$).

that the distribution of the lyso-PC peak coincided with the vanadate-sensitive ATPase (data not shown). SDS-PAGE analysis of various fractions (Fig. 3), showed that microsomal and plasma

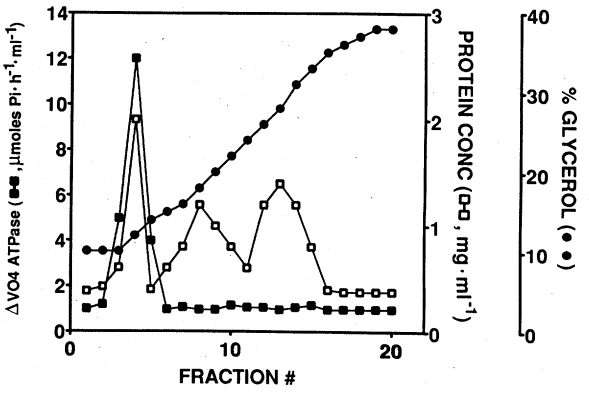

Fig. 2. Glycerol gradient of Lyso-PC solubilized plasma membrane. Lyso-PC solubilized proteins were loaded on a 10—40% (w/v) glycerol gradient and centrifuged at 32 000 rev./min overnight as described under Materials and Methods. Percent of glycerol (●—●), vanadate sensitive ATPase (■—■) and protein (□—□).

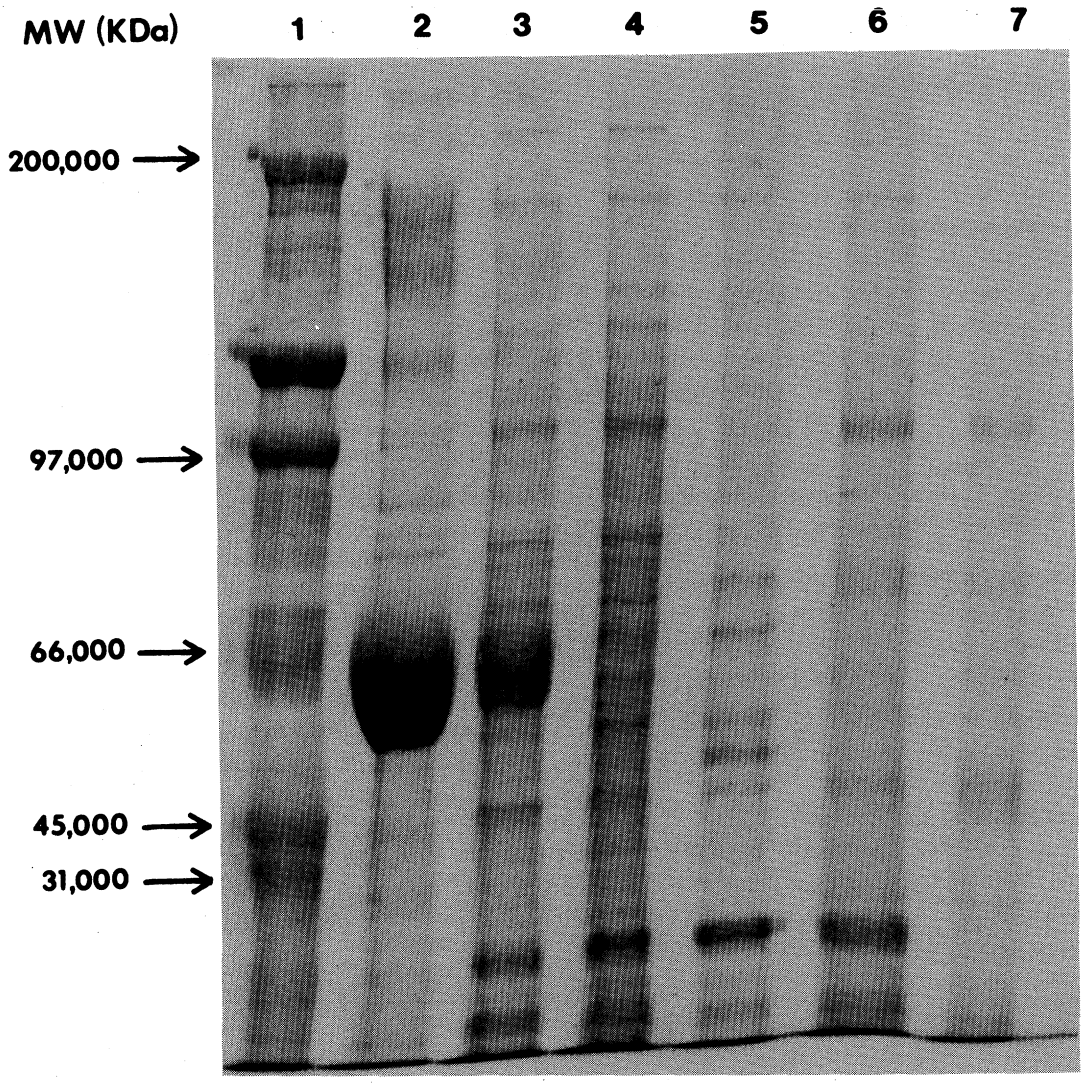

Fig. 3. SDS-PAGE analysis of various fractions during purification of plasma membrane ATPase. Proteins from various fractions were subjected to SDS-PAGE and stained with coomassie blue as described in Materials and Methods. Lane 1, molecular weight standards; 2, crude microsome; 3, KI-washed microsome; 4, plasma membrane; 5, pellet from lyso-PC solubilization; 6, supernatant fraction from lyso-PC solubilization; 7, fractions with ATPase activity from glycerol gradient.

membrane fractions (lanes 2 and 4) had a similar protein pattern, except that the latter contained more 100-kDa polypeptide and less BSA contamination. The lyso-PC procedure effectively solubilized all of the 100-kDa polypeptide into the supernatant fraction (lane 6) since this band was absent from the resulting pellet (lane 5). The fraction containing the vanadate-sensitive ATPase peak (lane 7) showed that most of the low molecular weight impurities were removed.

Table I summarizes the extent of purification. The specific activity of the highly purified ATPase (from the glycerol gradient) was 186 μmol h^{-1} mg (prot.)$^{-1}$. Lyso-PC solubilization significantly in-

Table I. Purification of plasma membrane ATPase from maize roots.

Fractions	Proteins[b]	Vanadate sensitive ATPase activity[a]	
		Total	Spec. act.[c]
Crude microsome	145	5191	35.8
Plasma membrane	43.5	1957	45.0
Solubilized ATPase	10.2	1672	164
Glycerol gradient	5.5	1015	186

[a]Vanadate sensitive ATPase activity was assayed as described in Materials and Methods. The activity is expressed as μmol/h.
[b]mg protein.
[c]Specific activity (μmol/h mg (prot.)$^{-1}$).

creased the specific activity of the ATPase but further glycerol gradient centrifugation did not significantly increase the specific activity.

Properties of antiserum to the ATPase

To prepare the ATPase antibody, partially purified enzyme from the glycerol gradient was subjected to SDS-PAGE. The band corresponding to the 100-kDa polypeptide was used as the antigen. ELISA, immunotitrations and immunoblots were performed to verify the specificity of the antibody. With a fixed amount of the antiserum, the extent of binding with different membranes isolated by the discontinuous sucrose gradient is shown in Table II. Using an undiluted antigen preparation, mitochondrial protein (approx. 0.1 mg membrane protein) showed a 33% cross reaction with the antiserum and the tonoplast fraction showed a 47% cross-reaction. As the antigen concentration was decreased, the cross-reactivity decreased. At a 1:20 antigen dilution, cross-reactivity with mitochondria and tonoplast was reduced to 1 and 13%, respectively, while plasma membrane retained more than 40% of its reactivity toward the antiserum. Thus, the polyclonal antiserum showed a higher specificity towards the plasma membrane.

Table II. Determination of the specificity of the antigen.

Antigen dilution	Protein source		
	Plasma membrane[a]	Tonoplast[a]	Mitochondria[a]
Undiluted	0.798 (100%)	0.376 (47%)	0.259 (33%)
1:10 dilution	0.646 (83%)	0.201 (25%)	0.110 (14%)
1:20 dilution	0.366 (46%)	0.105 (13%)	0.005 (0.6%)

[a]Subcellular fractions were obtained from discontinuous sucrose gradients. Tonoplast was obtained from the 28/34% interface. Plasma membrane was obtained from the 34/40% interface and mitochondria was obtained from the pellet. Protein concentrations for the undiluted antigens were 1 mg/ml. The reaction of antigen and antibody was determined by the ELISA method as described in Materials and Methods and the absorbance change at 415 nm was obtained by the addition of substrate (ABTS, 1 mM). The binding value of undiluted plasma membrane with antiserum was assigned a value of 100%.

To test further the antiserum specificity, a constant amount of lyso-PC solubilized enzyme was incubated with increasing amounts of antiserum. After incubation, the mixture was centrifuged to separate the antigen-antibody complex from free antigen. ATPase activities in both supernatant and pellet fractions were determined (Fig. 4). As the

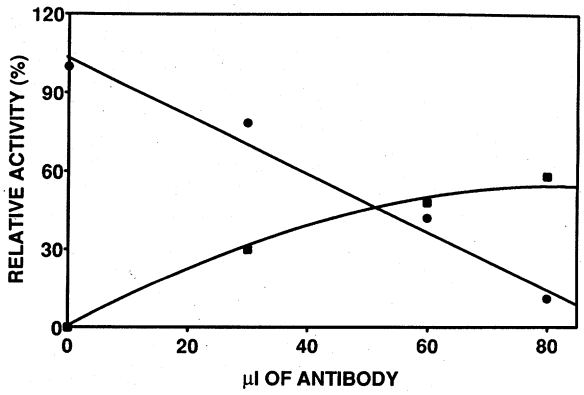

Fig. 4. Immunoprecipitation of ATPase lyso-PC solubilized proteins were precipitated by antiserum against plasma ATPase as described under Materials and Methods. ATPase activity in pellet (■ — ■) and supernatant (● — ●) fractions were assayed as described in Materials and Methods. 100% activity = 160 μmol P_i h^{-1} mg (prot.)$^{-1}$.

concentration of antiserum was increased, the ATPase activity in the supernatant fraction decreased, while the ATPase activity in the pellet fraction increased. Thus, increasing the antibody concentration resulted in removal of the ATPase and this activity was recovered in the pellet fraction. At 80 μl of antiserum, only 65% of the total ATPase activity was recovered from the supernatant and pellet fractions. This loss of activity could have resulted from direct inhibition of the ATPase by the antibody.

In order to verify that the specificity of the antibody was to the 100-kDa polypeptide of the plasma membrane, immunoblotting was also performed by transferring microsomal and plasma membrane proteins separated by SDS-PAGE to nitrocellulose (Fig. 5). The immunospot was detected by incubating the primary antigen-antiserum complex with anti-rabbit IgG conjugated with colloidal gold [18]. Staining with Amido Black of plasma membrane proteins showed a mixture of proteins, while immunogold staining (lanes 3 and 4) indicated only one band with a molecular weight of 100 kDa. Thus, the polyclonal antibody reported here was specific for the 100-kDa polypeptide of the plasma membrane ATPase.

To test whether the antibody which was generated is able to affect proton transport of the ATPase, reconstituted corn root plasma membrane vesicles were incubated with various concentrations of antiserum at room temperature for 2 h. Coupled proton pumping and ATP hydrolysis were measured. Table III shows that proton transport activity and ATP hydrolysis decreased as the concentrations of antibody increased. However, proton transport activity was inhibited to a greater extent at each level of antibody. For example, when proton transport activity was reduced by 50%, ATP hydrolysis activity was unchanged. When proton transport activity was completely inhibited, at least 20% of the original ATP hydrolysis activity remained.

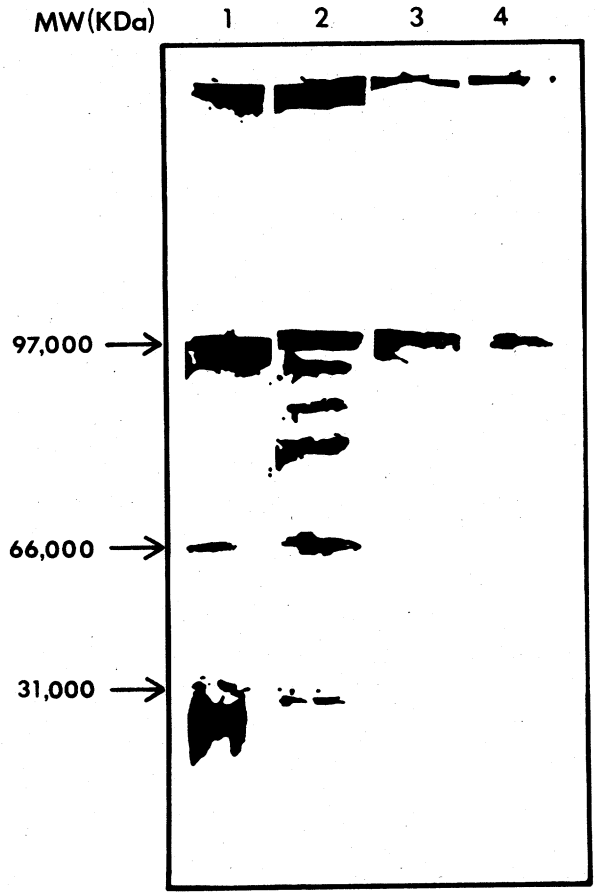

Fig. 5. Western blot transfer of various protein fractions from maize roots. After SDS-PAGE, proteins were transferred to nitrocellulose paper as described in Materials and Methods. Lane 1 (high molecular weight standards) and lane 2 (plasma membrane) were stained with ami 15 amido black, and lane 3 (KI-washed microsome) and lane 4 (plasma membrane) were stained with immuno-gold as described in Materials and Methods.

Discussion

As part of our ongoing research program to study the function of corn root plasma membrane ATPase, we sought to purify the ATPase and raise antibodies. To initiate this endeavour we chose to adopt the purification scheme used to isolate *Neurospora* plasma membrane ATPase [7]. This protocol involves removal of contaminants by washing the membranes with DOC and then solubilizing the ATPase with lyso-PC. The final purification step utilizes a glycerol gradient to obtain the ATPase in a state of high purity as an

Table III. The effect of anti-ATPase antiserum on the proton pumping and ATP hydrolysis of reconstituted plasma membrane ATPase. 125 μl of reconstituted protein was mixed with various concentrations of antiserum and the mixture was incubated at room temperature for 2 h. Proton pumping was assayed by AO as described in Materials and Methods. After the proton pumping reached a steady state, a 100-μl aliquot from the reaction mixture was assayed for P_i released as described in Materials and Methods.

	ATP hydrolysis (total μmol of P_i)	Proton pumping ΔA492 nm/min)
Control	258 (100%)	0.0020 (100%)
25 μl antiserum	256 (100%)	0.0020 (100%)
50 μl antiserum	282 (100%)	0.0013 (65%)
75 μl antiserum	284 (100%)	0.0010 (50%)
100 μl antiserum	50 (20%)	0.0000 (0%)

oligomer of 6 copies of the 105 000-Da subunit [7]. The specific activity of the corn root ATPase in the membrane pellet was increased by extraction of the vesicles with DOC. The optimum concentration of DOC was between 0.1 and 0.2% (w/v). This level is far lower than the 0.6% used for *Neurospora*, but comparable to that reported by Gallagher and Leonard [19]. The ATPase in the DOC-treated membrane was readily solubilized by lyso-PC. As in the case of *Neurospora*, solubilization of active ATPase reqired the presence of its substrate Mg:ATP and the inhibitor vanadate. The effectiveness of the three agents in maintaining ATPase activity is believed to involve the formation of an intermediate conformation that is resistant to denaturation and proteolytic cleavage [13]. It should be mentioned that this vanadate concentration (the final concentration of vanadate in Fig. 1 was less than 0.1 mM) did not interfere with the assay of ATPase activity, because of dilution and presence of EDTA in the assay buffer [13].

When the lyso-PC solubilized ATPase from *Neurospora crassa* was subjected to glycerol gradient centrifugation, a significant degree of purification was achieved [7]. The ATPase sedimented to about 35% (w/v) glycerol due to the formation of a complex consisting of 6 copies of the 105 000-Da subunit [20]. The behavior of the corn root enzyme was very different from that of *Neurospora*. The ATPase apparentiy did not enter the gradient, indicating that aggregation had not occurred. When the fraction containing the highest specific activity of ATPase activity from the glycerol gradient was subjected to gel filtration on a Sephadex G-100 column, the ATPase activity eluted with the retention volume indicating an apparent molecular weight of 100 000 (unpublished data). These results indicate that the corn root ATPase was solubilized by lyso-PC in a monomeric form and the monomeric form is capable of catalyzing ATP hydrolysis.

Solubilization of the corn root enzyme in an apparent monomeric form is also unique when compared to the purification of other higher plant plasma membrane ATPases. Based on density centrifugation and gel filtration experiments, red beet ATPase exists as a high molecular weight aggregate when solubilized with either octylglucoside [8] or zwittergent 3-14 [21]. In addition, the ATPase from corn roots and oat also appear as high molecular weight aggregates when solubilized by lyso-PC [22]. The reason for the discrepancy between this report and previous observations remains to be established. Recently, Briskin and Reynold-Niesman [21] reported that the red beet plasma membrane ATPase could be solubilized in a monomeric form based on radiation inactivation. However, the use of radiation inactivation to determine the molecular weight of membrane proteins with complex reaction cycles, like transporters, has recentiy been questioned [23].

The activity of the lyso-PC solubilized and glycerol gradient purified ATPase was not stimulated by the addition of other lipids including bovine brain folch fraction I and asolectin (unpublished data). Analysis of the glycerol gradient fractions after extraction with organic solvents and TLC revealed the presence of a high amount of lyso-PC. These results are in agreement with earlier findings from our laboratory that the phosphocholine head group is able to activate the ATPase [24].

We separated the proteins of purified corn root plasma membrane by SDS-PAGE and used the 100-kDa subunit as the antigen source to raise antibodies. Evidence has been presented that the 100-

kDa polypeptide contains the active site of the ATPase [25]. ELISA data indicated that the antibody is quite specific for the plasma membrane, but some nonspecific binding to other subcellular membranes was also detected. Western-blotting data indicated that the antiserum was specific for the 100-kDa polypeptide for the plasma membrane ATPase.

Upon immunoprecipitation (Fig. 4), the ATPase activity in the supernatant fraction was reduced by 90%. However, 65% of the activity before immunoprecipitation was recovered in the pellet fraction. Therefore, only 25% of the activity was inhibited by immunoprecipitation. This 25% of the activity lost could have resulted from the direct inhibition of the enzyme by the antibody. These data suggest that the immunogenic binding sites of the ATPase may be different from the catalytic site of the enzyme, since inhibition was less than the percentage precipitated by the antibody.

The results in Table III indicate that when the antibody reacted with reconstituted vesicles from corn root plasma membrane, the proton pumping activity of reconstituted vesicles was more sensitive to the presence of antiserum than ATP hydrolysis. This result suggests that the coupling of proton pumping and ATP hydrolysis in the plasma membrane may be indirect in nature, similar to the findings of Tu et al. [17] for tonoplast ATPase. Further experiments need to be conducted to confirm this indirect coupling mechanism of proton transport and ATP hydrolysis in the plasma membrane.

References

1 H. Sze, H^+-translocating advances using membrane vesicles. Annu. Rev. Plant Physiol., 36 (1985) 175—208.
2 R. Serrano, Plasma membrane ATPase of fungi and plants as a novel type of proton pump. Curr. Top Cell Res., 23 (1984) 87—121.
3 M.I. De Michelis and R.M. Spanswick, H^+-pumping driven by the vanadate sensitive ATPase in membrane vesicles from corn roots. Plant Physiol., 81 (1986) 542—547.
4 J.L. Giannini, L.H. Gildensoph and D.P. Briskin, Selective production of sealed plasma membrane vesicles from red beet (*Beta vulgaris* L.) storage tissue. Arch. Biochem. Biophys., 254 (1987) 621—630.
5 R. Serrano, Purification of the proton-pumping ATPase from plant plasma membranes, Biochem. Biophys. Res. Commun., 121 (1984) 735—740.
6 M.J. Benson and C.L. Tipton, Purification and characterization of cation-stimulated adenosine triphosphatase from corn roots. Plant Physiol., 62 (1978) 165—172.
7 R. Smith and G.A. Scarborough, Large-scale isolation of the Neurospora plasma membrane H^+-ATPase. Anal. Biochem., 138 (1984) 156—163.
8 F.M. DuPont and R.T. Leonard, Solubilization and purification of adenosine triphosphatase from a corn root plasma membrane fraction. Plant Physiol., 65 (1980) 931—938.
9 D.P. Briskin and R.J. Poole, Characterization of the solubilized ATPase from red beets. Plant Physiol., 76 (1984) 26—30.
10 T. Nagao, H. Sasakawa and T. Sugiyama, Purification of H^+-ATPase from the plasma membrane of maize roots and preparation of its antibody. Plant Cell Physiol., 28 (1987) 1181—1186.
11 F.M. DuPont, C.K. Tanaka and W.J. Harkman, Separation and immunological characterization of membrane fractions from barley roots. Plant Physiol., 86 (1988) 717—724.
12 D. Brauer, A.-F. Hsu and S.-I Tu, Factors associated with instability of proton pumping by maize root microsomes. Plant Physiol., 87 (1988) 598—602.
13 R. Addison and G.A. Scarborough, Solubilization and purification of the *Neurospora* plasma membrane H^+-ATPase. J. Biol. Chem., 256 (1981) 13165—13171.
14 B.N. Ames, Assay of inorganic phosphate, total phosphate and phosphatase. Methods Enzymol., 8 (1966) 115—118.
15 A. Bensadoun and D. Weinstein, Assay of proteins in the presence of interferring materials. Anal. Biochem., 70 (1976) 241—250.
16 B. Clark and E. Engvalle, Enzyme linked immunosorbent assay (ELISA), in: E.T. Maggio (Ed.), Enzyme Immunoassay, Chapter 8, CRC Press, 1986, pp. 167—196.
17 S.-I. Tu, G. Nagahashi and J.N. Brouillette, Proton pumping kinetics and origin of nitrate inhibition of tonoplast-type H^+-ATPase. Arch. Biochem. Biophys., 256 (1987) 625—637.
18 Y.H. Hsu, Immunogold for detection of antigen on nitrocellulose paper. Anal. Biol., 142 (1984) 221—225.
19 S.R. Gallagher and R.T. Leonard, Electrophoretic characterization of a detergent-treated plasma membrane fraction from corn roots. Plant Physiol., 83 (1987) 265—271.
20 C.C. Chadwick, E. Goormaghtigh and G.A. Scarborough, A hexameric form of *Neurospora crassa* plasma membrane H^+-ATPase. Arch. Biochem. Biophys., 252 (1987) 348—356.
21 D.B. Briskin and I. Reynolds-Niesman, Change in target molecular size of the red beet plasma membrane ATPase during solubilization and reconstitution. Plant Physiol., 90 (1989) 394—397.

22 N.E. Balke and T.K. Hodges, Plasma membrane adenosine triphosphatase of oat roots. Plant Physiol., 55 (1975) 83—86.

23 S.G. McGrew, M. Inui, C.C. Chadwick, R.J. Boucek, Jr., C.Y. Jung and S. Fleischer, Comparison of the calcium release channel of cardiac and skeletal muscle sarcoplasmic by reticulum target inactivation analysis. Biochem., 28 (1989) 1319—1323.

24 D. Brauer and S.-I. Tu, Phospholipid requirement of the vanadate-sensitive ATPase from maize roots evaluated by two methods. Plant Physiol., 89 (1989) 867—874.

25 E. Goormaghtigh, C. Chadwick and G.A. Scarborough, Monomers of the *Neurospora* plasma membrane H^+-ATPase catalyze efficient proton translocation. J. Biol. Chem., 261 (1986) 7466—7471.